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Original article

Novel 1,4-benzoxazine and 1,4-benzodioxine inhibitors of angiogenesis

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ABSTRACT

Esters of 1,4-benzoxazine and 1,4-benzodioxine compounds **1** and **10**, which combine thrombin inhibitory and GPIIb/IIIa antagonistic activity in one molecule are shown to inhibit endothelial cell migration and tube formation *in vitro* and angiogenesis in the chicken chorioallantoic membrane (CAM) assay. The corresponding carboxylic acids **1** ($R^2 = H$) and **11** were devoid of anti-angiogenic activity, most probably due to their insufficient entry into the cell. Although thrombin inhibition remains the most probable explanation for their inhibition of angiogenesis, VEGFR2 kinase assay suggest that other targets such as VEGFR2 might be involved.

1. Introduction

The association of venous thrombosis and cancer has been recognized for over 100 years and has a prevalence rate of 10–20% [1]. A systemic activation of blood coagulation which leads to increased tendency toward formation of blood clots is frequently present in cancer patients. Most tumor cells have constitutively active tissue factor on their surface, capable of generating thrombin in plasma. The presence of thrombin has been shown in a variety of tumor types and a clinical study demonstrated that primary thromboembolism increases the risk of overt cancer diagnosis by 3-fold within 6–12 months after thrombosis [2]. These clinical observations are in line with animal experiments where thrombin treatment of B16 melanoma tumors increases dramatically the number of lung metastases in rats [3]. Malignancy initiates a vicious cycle in which greater tumor burden supplies more thrombin that stimulates tumor growth and increases platelet-tumor interaction. The tumor-promoting effects of thrombin may be related to its pro-angiogenic activity, which is thought to be mediated by activation of its protease-activated receptor (PAR-1) which leads to downstream mitogenic signaling events resulting *inter alia* in the expression of vascular endothelial growth factor (VEGF) in tumor cells and its tyrosine kinase receptor VEGFR2 in endothelial cells [4–6].

Thrombin stimulates the migration of tumor cells into the vasculature and, together with other tumor secreted agents, activates the endothelial cells and platelets to expose P-selectin. Weakly activated platelets and endothelial cells bind tumor cells via P-selectin exposed on their surface inducing weak tethering of tumor cells to the endothelium and platelets. Finally, a firm binding of tumor cells to platelets occurs through interaction mediated by binding of platelet integrin GPIIb/IIIa to tumor integrins via RGD motif-containing ligands, such as von Willebrand Factor (vWF) and fibronectin. These events lead to angiogenesis via thrombin-stimulated synthesis and release of VEGF and other proangiogenic growth factors from tumor cells and platelets and induction of VEGFR2 synthesis in endothelial cells. Platelet-tumor aggregates protect tumor cells from natural killer cells, prolong their survival in the blood and bind more avidly to subendothelial basement membranes and matrix. Many tumor cells require platelets for the development of metastasis and it has been shown that several tumor cell lines aggregate platelets *in vitro* [4,5,8]. Targeting the aberrant growth of blood vessels, a common biological aspect of anti-angiogenic drugs [9–11], is extensively being explored in oncology in order to deprive tumors of nutrients normally delivered by blood flow [12–15]. Recent studies indicate that

Abbreviations: BAEC, bovine aortic endothelial cells; CAM, chick chorioallantoic membrane; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; DIAD, diisopropyl azodicarboxylate; GPIIb/IIIa, glycoprotein IIb/IIIa; HMEC, human microvascular endothelial cells; MAEC, mouse aortic endothelial cells; MCF-7, a human breast cancer cell line; VEGFR2, vascular endothelial growth factor receptor 2.

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angiogenesis inhibitors, by depriving tumors of oxygen, can have an unintended effect – promotion of metastasis [16–20].

Both thrombin and integrin GPIIb/IIIa are thus important players in angiogenesis and metastasis. The thrombin inhibitor hirudin was demonstrated to inhibit angiogenesis in a chick chorioallantoic membrane assay [7] and in some models RGD-containing peptides were shown to block metastasis [5]. We have recently described novel potential dual antithrombotic compounds which comprise in the same molecule both thrombin inhibitory and fibrinogen receptor (GPIIb/IIIa) antagonistic activity due to highly overlapped thrombin inhibitor and fibrinogen receptor antagonist pharmacophores [21–23]. Knowing the interplay between cancer and thrombosis, with thrombin and platelet GPIIb/IIIa receptor as key players involved in angiogenesis and metastasis, we wanted to investigate whether our compounds with thrombin inhibitory and GPIIb/IIIa antagonistic activity are endowed with antiangiogenic activity. Small-molecule multitarget compounds with antithrombotic, antiangiogenic and possible antimetastatic activity would present an interesting synergistic approach in cancer therapy which has also been reported for phosphomannopentaose sulfate (PI-88), a multi-component mixture of phosphomannopentaose and phosphomannotetraose sulfates and related heparan sulfate mimetics [24]. The sulfated oligosaccharide PI-88 is a potent antiangiogenic and antimetastatic agent which also inhibits thrombin but does not aggregate platelets [24,25]. In this paper we (i) report on the antiangiogenic activity of two series of our multitarget compounds combining in the same molecule highly overlapped pharmacophores of thrombin inhibitors and GPIIb/IIIa antagonists and (ii) seek to establish a rough structure–activity relationship. and (iii) discuss a possible mechanism responsible for their inhibition of angiogenesis.

2. Results and discussion

2.1. Chemistry

The design and synthesis of 1,4-benzoxazine compounds represented by general structures **1a** and **1b** has been described recently [22,23]. They comprise highly integrated pharmacophores of thrombin inhibitors (a P_1 benzamidine group, a P_2 benzoxazine core and P_3 *N*-carboxymethyl-benzylamino or *N*-oxalyl-benzylamino moieties) and GPIIb/IIIa antagonists (a benzamidine moiety separated by a 2-hydroxymethyl-6/7-methylamino-1,4-benzoxazine spacer from a carboxylate group). The preparation of nitriles **2a**,**b** [22,23] and [1,2,4]triazolo[4,3,*b*]pyridazine analogs **2c** [26] has also been described (Fig. 1). The synthesis of 1,4-benzodioxine analogs **10a**,**b** and **11a**,**b** is presented in Schemes 1 and 2. The reaction of 4-nitrocatechol (**3**) with epichlorohydrin (**4**) in the presence of sodium hydrogen carbonate in *N*,*N*-dimethylformamide according to a published procedure [27] afforded (7-nitro-2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)methanol (**5b**) whereas the reaction of **3** with epichlorohydrin (**4**) using sodium hydride as a base gave the 6-nitro isomer **5a** (Scheme 1). Both nitro isomers were reacted with 4-hydroxybenzonitrile under Mitsunobu conditions to give ethers **6a** and **6b** which were reduced in the next step to amines **7a** and **7b** using catalytic hydrogenation over palladium on charcoal. The amines were benzylated using benzaldehyde and sodium borohydride and the resulting *N*-benzylamines **8a** and **8b** acylated with ethyl oxalyl chloride to give compounds **9a** and **9b**. They afforded amidines **10a**,**b** upon Pinner reaction, the ester group of which was hydrolyzed to the carboxylic acids **11a** and **11b** (Scheme 2).

The preparation of compounds **17a** and **17b**, lacking the basic benzamidine moiety is presented in Scheme 3. The 2-(hydroxymethyl)-2*H*-benzo[*b*][1,4]oxazine derivative **12** [22] was acetylated with acetic anhydride to give ester **13**, which upon catalytic reduction to amine **14** and further benzylation with benzaldehyde or 3,5-difluorobenzaldehyde afforded *N*-benzylamines **15a** and **15b**. These were acylated with ethyl oxalyl chloride to give *N*-ethyl oxalyl derivatives **16a** and **16b** which were finally hydrolyzed to afford carboxylic acids **17a** and **17b**.

2.2. Pharmacology

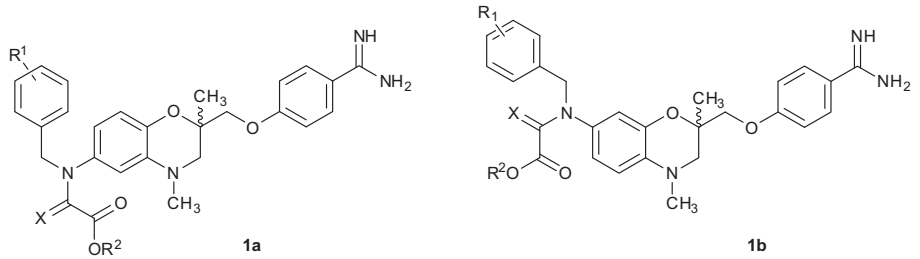
2.2.1. Inhibition of cell proliferation

Several *in vitro* and *in vivo* assays have been developed that recapitulate different steps of the angiogenesis process, including endothelial cell proliferation, migration and tube formation [28]. We first investigated the anti-proliferative activity of 1,4-benzoxazine compounds **1a** and **1b**, nitriles **2a** and **2b** [1,2,4],triazolo[4,3,*b*]pyridazines **2c**, 1,4-benzodioxines **10** and **11**, as well as compounds **16** and **17** lacking a basic P_1 moiety, in two endothelial cell lines [human microvascular endothelial cells (HMEC-1) and bovine aortic endothelial cells (BAEC)] [29,30]. The results collected in Table 1 demonstrate that esters **1a** and **1b** inhibit the proliferation of both endothelial cell lines equally well, with IC_{50} values of 7-*N*-alkylamino compounds **1b1**–**1b4** ranging from 1.8 to 4.1 μM and from 4.6 to 7.9 micromolar for 7-*N*-acylamino compounds **1b5**–**1b8**. Also the 6-substituted compounds **1a1**–**1a8** showed anti-proliferative activity with a trend toward more pronounced cytostatic activity of 6-*N*-alkylamino compounds **1a1**–**1a4** (IC_{50} ranging from 3.8 to 6.7 μM) versus 6-*N*-acylamino compounds **1a5**–**1a8** (IC_{50} ranging from 6.6 to 17.9 μM). In both endothelial cell lines the *N*-acylamino-1,4-benzodioxine compounds (*S*)-**10a** and **10b** were found to be about 3-fold weaker inhibitors of cell proliferation than the corresponding 1,4-benzoxazine compounds.

[1,2,4]Triazolo[4,3-*b*]pyridazine compounds **2c** (R^2 = Et) lacking a basic benzamidine moiety inhibited proliferation of BAEC and HMEC-1 (IC_{50} values between 33.5 and 46.0 μM; results not shown) although they were found to be up to 10-fold less potent than amidines **1a** and **1b**. The tested 6-and 7-substituted nitriles **2a** and **2b**, showing a high similarity to amidines **1a** and **1b**, were either inactive (i.e. compounds **2a1** and **2a4**) or weakly active (i.e. compound **2b4**) with IC_{50} values in the range of 24.6–49.7 μM. Compounds **16a** and **16b**, lacking the basic benzamidino moiety as well, also displayed no (i.e. compound **16a**) or a weak (i.e. compound **16b**) inhibition of HMEC-1 and BAEC proliferation (IC_{50} values of 89.4 and 59.6 μM respectively), supporting the view that in the tested series of compounds a benzamidine moiety is important for antiproliferative activity. All tested carboxylic acids series (**1a** and **1b**, **2c**: R^2 = H; **11a** and **11b**; **17a** and **17b**) were devoid of anti-proliferative activity (IC_{50} > 100 μM; results not shown), suggesting that cellular uptake, which may be significantly hampered in zwitterionic compounds, is required for anti-proliferative activity.

Next, all compounds were evaluated for their capacity to inhibit the proliferation of two carcinoma cell lines [human cervical carcinoma cells (HELA) and human breast carcinoma cells (MCF-7)] (Table 1). The compounds showed comparable anti-proliferative activity in tumor cells and endothelial cells, the most active compounds being 6-*N*-alkylamino compounds **1a1**–**1a4** and 7-*N*-alkylamino compounds **1b1**–**1b4** with IC_{50} values between 3.5 and 5.3 μM. These data indicate that the compounds show no selectivity toward any of the tested cell types.

Several compounds not only inhibited cell growth (i.e. cytostatic action) but, at a higher concentration, also induced cell death (*i.e.* cytotoxic action). In particular, all 1,4-benzoxazine compounds were toxic at 100 μM after 3 days in culture (not shown). Both 6-and 7-*N*-alkylamino series **1a1**–**1a4** and **1b1**–**1b4** were still toxic at 30 μM, whereas the respective *N*-acylamino series **1a5**–**1a8** and **1b5**–**1b8** displayed no toxicity at this



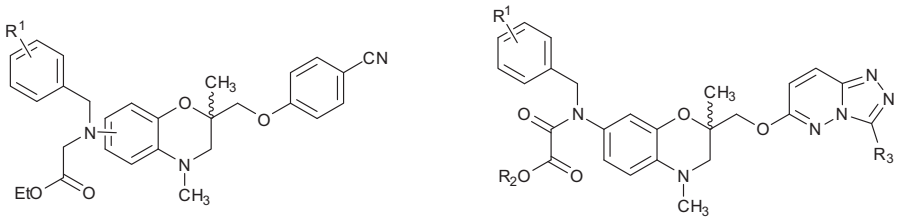


Fig. 1. 1,4-Benzoxazines **1a**, **1b** with thrombin inhibitory and GPIIb/IIIa antagonistic activity, intermediary nitriles **2a** and **2b** and [1,2,4]triazolo[4,3,*b*]pyridazine analogs **2c**.

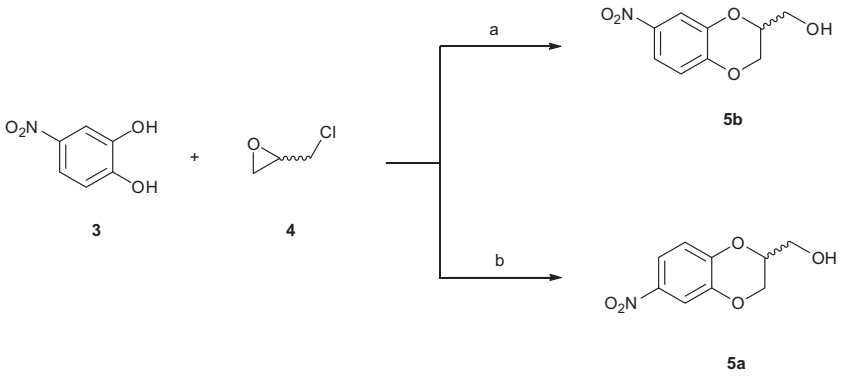
concentration. In contrast to benzoxazines **1a** and **1b** the benzodioxine compounds (*S*)-**10a** and **10b** were not toxic at 100 μM (not shown). These results highlight the contribution of the *N*-ethyl oxalyl substituent and 1,4-benzodioxine scaffold to lowering the toxicity of these compound series.

2.2.2. Inhibition of endothelial cell migration

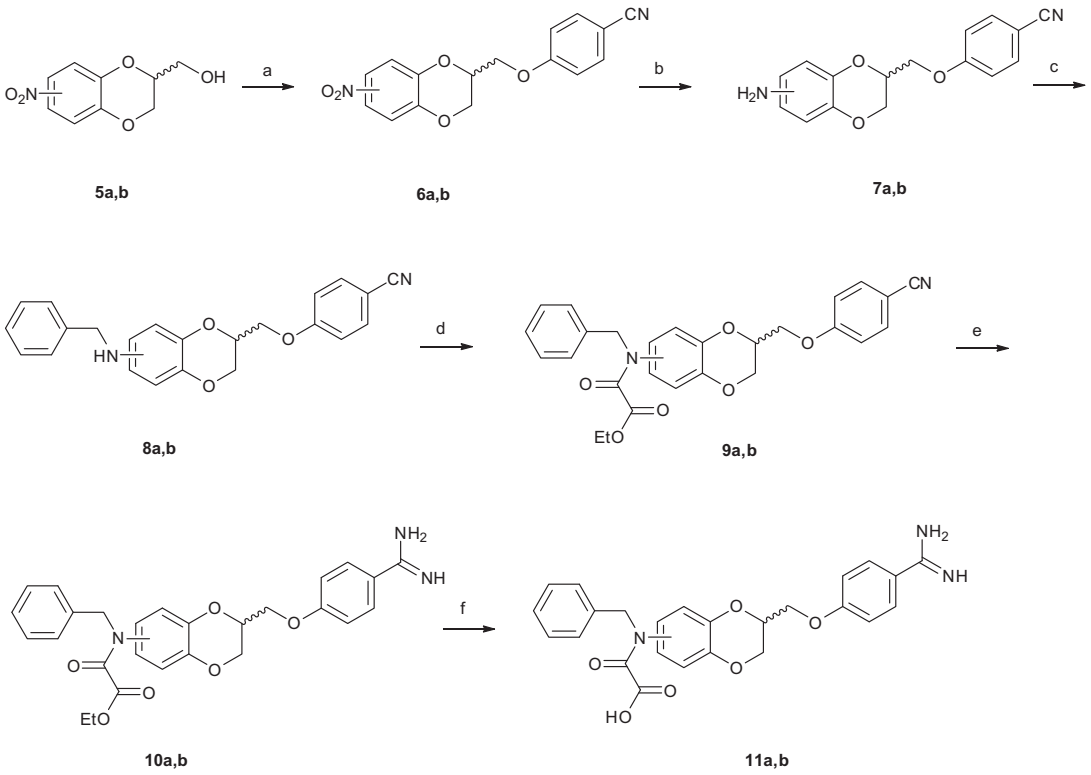
Endothelial cell migration is an essential step in angiogenesis. Therefore, compounds with anti-proliferative activity (i.e. 1,4-benzoxazines of **1a** and **1b** series, benzodioxine **10b**, compounds **16a** and **16b**) were tested for possible inhibition of endothelial cell migration in a wound closure assay [28,30]. As shown in Fig. 2, a clear dose-dependent inhibitory effect was observed for all compounds. Also here, there was a trend toward more pronounced inhibition of cell migration by the 6-*N*-alkylaminobenzoxazine **1a1**–**1a4** and 7-*N*-alkylaminobenzoxazine series **1b1**–**1b4** versus their acyl counterparts **1a5**–**1a8** and **1b5**–**1b8**. In particular, the *N*-alkyl compounds caused a complete (or nearly complete) inhibition of MAEC migration at 30 μM and still inhibited wound closure by about 50% at 10 μM. A higher than 40% inhibition of MAEC cell migration was still present at 3 μM concentration for **1a1**, **1a4**, **1a5**, **1b2**, and **1b8** (Fig. 2). The most potent inhibitor of endothelial cell migration was **1b2**, which showed 95%, 78% and 41% inhibition of cell migration at 30, 10, and 3 μM, respectively. Interestingly, compound **10b** of the 7-*N*-acylaminobenzodioxine series and compounds **16a** and **16b** without a basic benzamidine moiety, which showed only modest anti-proliferative activities, displayed a potent inhibition of MAEC migration at 30 μM (more than 80% inhibition) and 10 μM (more than 40% inhibition) (Fig. 2).

2.2.3. Inhibition of tube formation

One of the most specific tests for angiogenesis is the matrigel tube formation assay which measures the ability of endothelial cells to form three-dimensional structures (tubes) [30,31]. Among the compounds tested at 30 and 10 μM concentration, 6-*N*-alkylaminobenzoxazine derivatives **1a1**–**1a4** as well as 7-*N*-alkylaminobenzoxazine derivatives **1b1**–**1b4** completely inhibited tube formation at 30 μM and were weakly active or inactive at 10 μM. The corresponding *N*-acylamino compounds **1a5**–**1a8** and **1b5**–**1b8** as well as the 7-*N*-acylaminobenzodioxine derivative **10b**,



Scheme 1. Synthesis of the benzodioxine scaffold. Reagents and conditions: (a) $NaHCO_3$, DMF, 80 °C, 12 h, (b) NaH, DMF, 80 °C, 12 h.

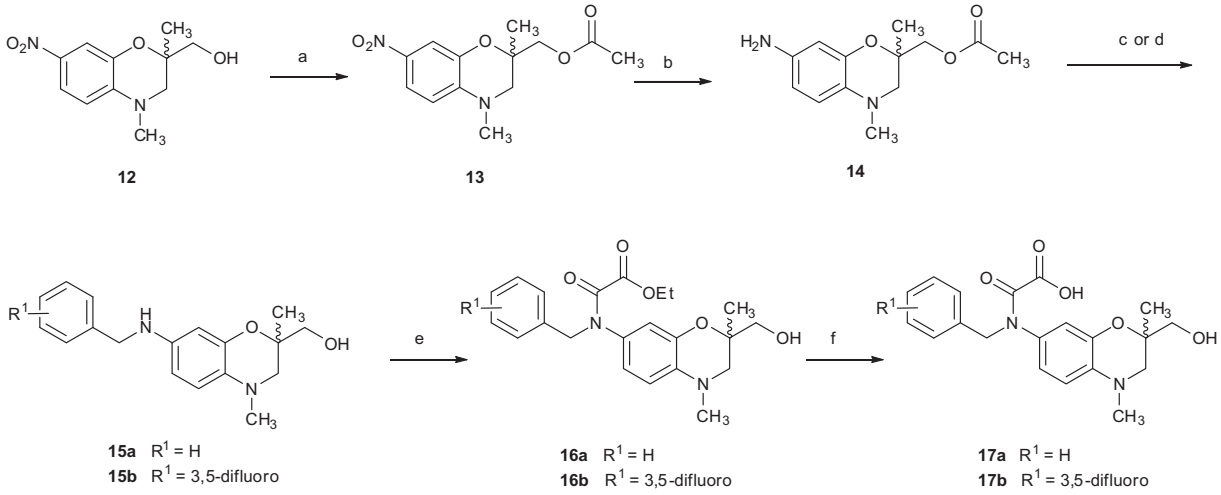


Scheme 2. Synthesis of target 1,4-benzoxazine compounds: (a) 4-cyanophenol, PPh_3, DIAD, THF, reflux, 48 h; (b) H_2, Pd/C, 25 bar, rt, 1 h; (c) benzaldehyde, MeOH, mol. sieves, rt, 12 h, then $NaBH_4$, 1 h; (d) ethyl oxalyl chloride, Et_3N, DCM, rt, 2 h; (e) HCl_g, EtOH, 0 °C, 30 min, rt, 24 h, then CH_3COONH_4, rt 24 h; (f) 1M LiOH, THF/MeOH, rt, 2 h.

being less toxic than the corresponding *N*-alkylamino compounds exhibited a concentration-dependent inhibition of tube formation. Interestingly, compounds **16a** and **16b**, lacking a basic benzamidino moiety also inhibited tube formation at 100 and 30 μM (Fig. 3).

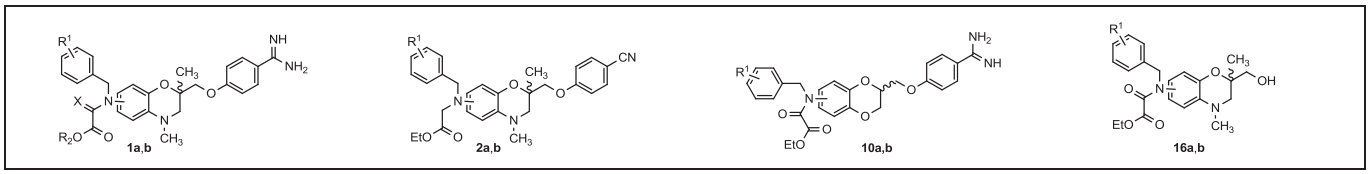
2.2.4. Inhibition of angiogenesis in the chick chorioallantoic membrane (CAM) assay

The CAM assay is an *in vivo* test in which potential inhibitors of angiogenesis are assessed by their effect on normal vascular development in chick embryos [28–30]. Both 6-and 7-*N*-alkylamino as well as 6-and 7-*N*-acylamino esters of the benzoxazine series (**1a**, **1b**; R_2 = Et) and *N*-acylamino esters of the of the benzodioxine series ((*S*)-**10a**, **10b**) were found to be potent inhibitors of angiogenesis in the CAM assay at 250 nmol/disc, while in the same experiment the corresponding carboxylic acids (**1a**, **1b**; R_2 = H and **11a**, **11b**) were devoid of angiogenesis inhibiting activity (data not shown). The observed inhibition of angiogenesis may be attributed to thrombin inhibition, since compounds **1a**, **1b**, (*S*)-**10a**, and **10b** are all moderate to potent thrombin inhibitors with K_i values in the range of 18 nM to 5.05 μM [23] and inhibition of angiogenesis in chick chorioallantoic membrane by the thrombin inhibitor hirudin has been reported [7]. Compounds of the carboxylic acids series



Scheme 3. Reagents and conditions: (a) acetic anhydride, 100 °C, 5 h; (b) H_2, Pd/C, 25 bar, rt, 1 h; (c) benzaldehyde, MeOH, mol. sieves, rt, 12 h, then $NaBH_4$, 1 h; (d) 3,5-difluorobenzaldehyde, MeOH, mol. sieves, rt, 12 h, then $NaBH_4$, 1 h; (e) ethyl oxalyl chloride, Et_3N, DCM, rt, 2 h; (f) 1M LiOH, THF/MeOH, rt, 2 h.

Table 1
Antiproliferative activity in HMEC-1, BAEC, HELA and MCF-7 cell lines. Mean ± SD are shown.



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Comp. No.	Subst. Position	R^1	X	R^2	HMEC-1 IC_{50} (μM)	BAEC IC_{50} (μM)	HELA IC_{50} (μM)	MCF-7 IC_{50} (μM)
1a1	6	3-F	H,H	Et	6.0 ± 0.1	3.9 ± 0.1	4.6 ± 0.8	4.2 ± 0.8
1a2	6	4-F	H,H	Et	6.0 ± 1.1	3.8 ± 0.5	4.8 ± 0.9	4.1 ± 0.6
1a3	6	3-F, 4-F	H,H	Et	4.2 ± 0.4	4.1 ± 0.3	4.8 ± 0.9	3.9 ± 0.7
1a4	6	3-F, 5-F	H,H	Et	6.7 ± 0.0	4.0 ± 0.2	4.5 ± 1.1	4.5 ± 0.3
1a5	6	3-F	O	Et	7.7 ± 1.1	18 ± 4	20 ± 4	6.5 ± 0.5
1a6	6	4-F	O	Et	8.4 ± 0.1	6.6 ± 1.4	14 ± 7	5.5 ± 0.8
1a7	6	3-F, 4-F	O	Et	8.3 ± 0.8	7.4 ± 1.6	14 ± 8	6.3 ± 1.0
1a8	6	3-F, 5-F	O	Et	7.8 ± 1.1	11 ± 6	6.9 ± 2.7	5.2 ± 1.1
1b1	7	3-F	H,H	Et	2.6 ± 0.1	4.1 ± 0.3	4.5 ± 0.5	3.9 ± 0.4
1b2	7	4-F	H,H	Et	2.9 ± 0.7	2.9 ± 0.8	4.0 ± 0.1	5.3 ± 0.2
1b3	7	3-F, 4-F	H,H	Et	2.5 ± 0.1	4.0 ± 0.7	4.1 ± 0.2	4.0 ± 0.3
1b4	7	3-F, 5-F	H,H	Et	1.8 ± 0.1	4.1 ± 0.8	4.9 ± 0.5	3.5 ± 0.9
1b5	7	3-F	O	Et	7.9 ± 0.6	6.5 ± 0.1	7.7 ± 0.3	3.8 ± 1.9
1b6	7	4-F	O	Et	7.6 ± 0.4	6.9 ± 0.5	14 ± 4	6.3 ± 0.6
1b7	7	3-F, 4-F	O	Et	6.7 ± 0.4	4.9 ± 1.0	11 ± 4	5.8 ± 0.8
1b8	7	3-F, 5-F	O	Et	6.8 ± 0.4	4.6 ± 0.3	6.6 ± 0.5	2.9 ± 0.4
2a1	6	3-F	–	–	>100	>100	>100	>100
2a4	6	3-F, 5-F	–	–	>100	>100	>100	>100
2b2	7	4-F	–	–	>100	50 ± 7	>100	>100
2b4	7	3-F, 5-F	–	–	49 ± 1	25 ± 9	>100	54 ± 4
10a(*S*)	6	H	–	–	24 ± 1	12 ± 0	19 ± 2	9.5 ± 2.3
10b	7	H	–	–	22 ± 3	8.5 ± 0.2	8.0 ± 3.1	8.7 ± 2.7
16a	7	H	–	–	>100	>100	>100	>100
16b	7	3-F, 5-F	–	–	89 ± 7	60 ± 9	44 ± 6	45 ± 4
SU5416	–	–	–	–	15 ± 9	8.3 ± 2.2	20 ± 6	1.9 ± 1.5

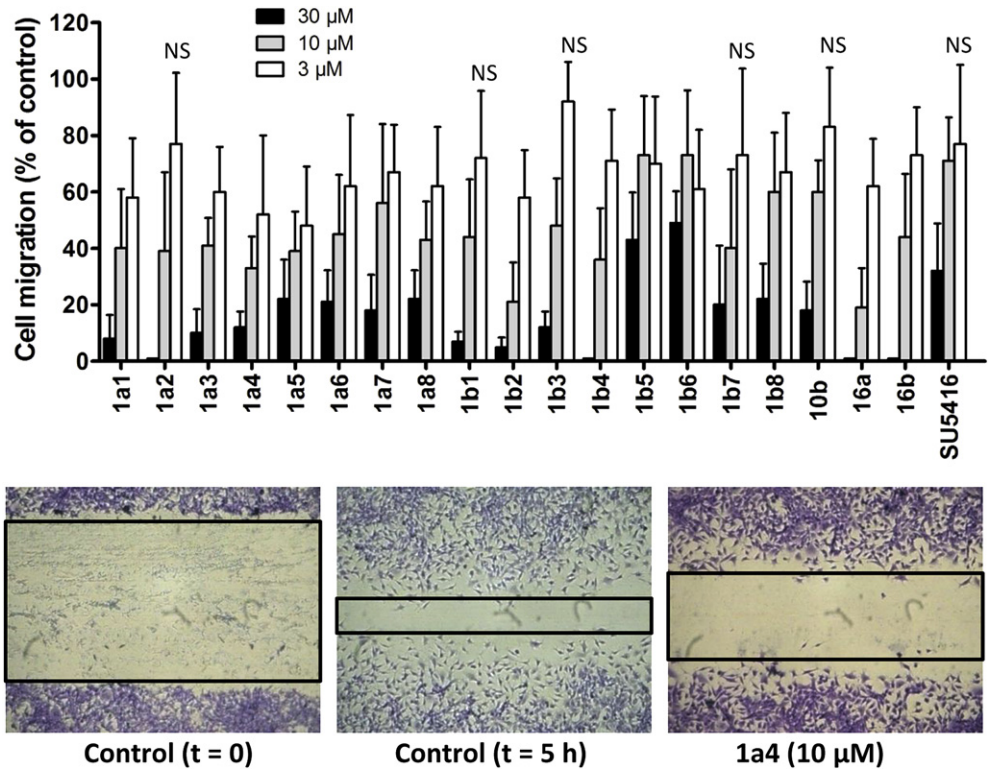

Fig. 2. MAEC wound closure (migration) assay; results are the mean (±SD) of 2–4 independent experiments. All data $p < 0.05$, except NS (not significant).

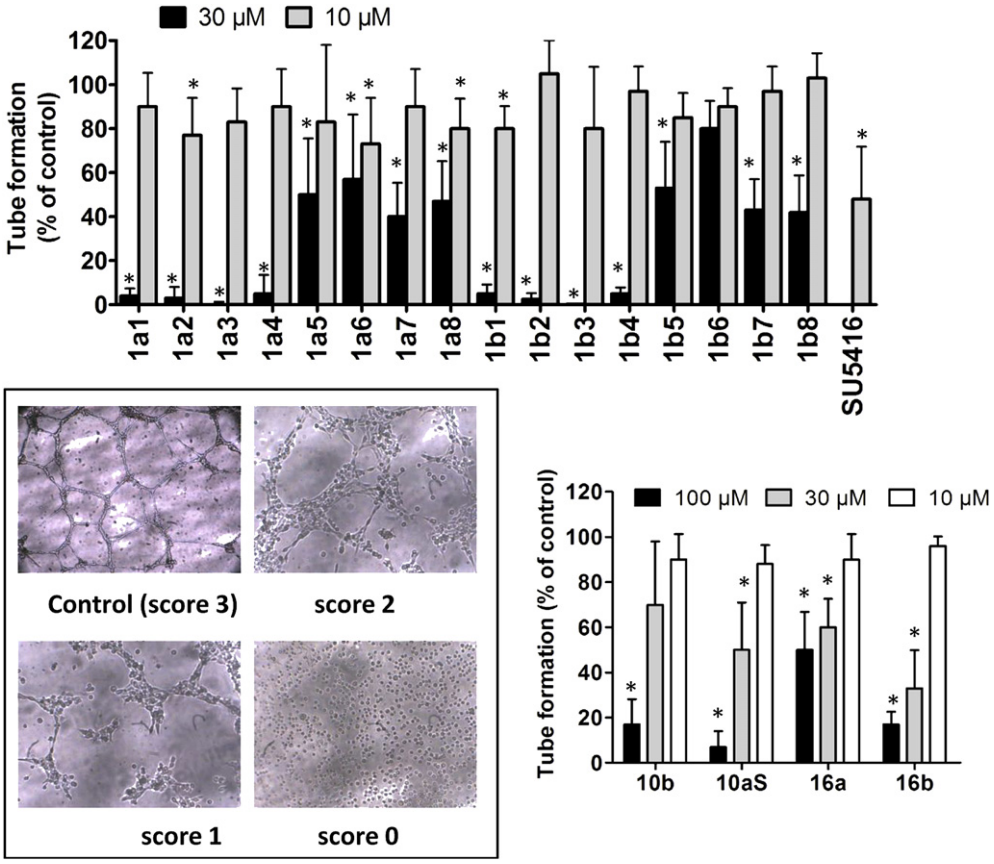

Fig. 3. Tube formation assay in matrigel. Tube formation was evaluated semi-quantitatively by giving a score from 0 to 3. Representative pictures are shown. Results are the mean (±SD) of 2–4 independent experiments. $^{*}p < 0.05$.

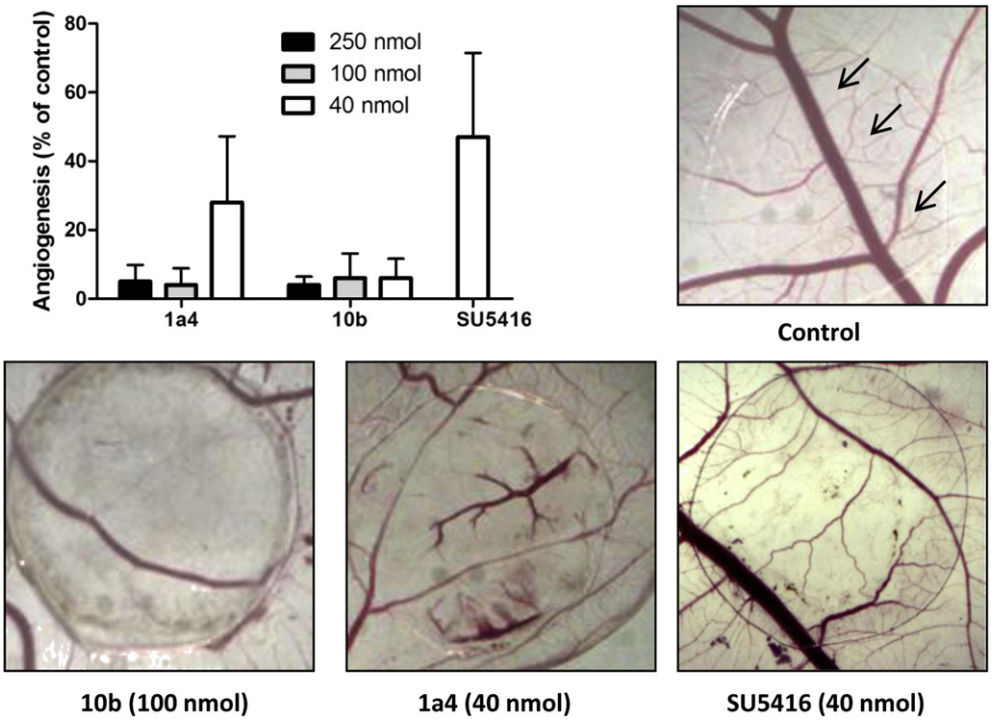

Fig. 4. Inhibition of angiogenesis in the CAM assay. At day 11, control CAMs are characterized by a network of blood vessels of different size. Only the capillaries (arrows) are formed during the course of treatment. At 250 and 100 nmol/disc, compounds **1a4** and **10b** cause a complete inhibition of angiogenesis and the disappearance (distruction) of the immature blood vessels, resulting in a nearly avascular CAM. Only major, pre-existing mature blood vessels are not affected by the compounds. At 40 nmol/disc, only nearly destructed, tortuous blood vessels can be seen with **1a4**, whereas **10b** still results in an avascular CAM (not shown). The VEGF antagonist SU5416 was toxic at 100 nmol, but inhibited the formation of new blood vessels at 40 nmol/disc. $n = 6–12$, $p < 0.05$. Mean ± SD are shown.

(**1a**, **1b**; R_2 = H and **11a**, **11b**), which are also thrombin inhibitors, although generally an order of magnitude less potent than the corresponding esters [23], were devoid of anti-angiogenic activity in the CAM assay (data not shown), again confirming that cell penetration, which is expected to be impaired in zwitterionic carboxylic acids, is required for biological activity of these compounds.

Two compounds of each series (i.e. **1a4** of the benzoxazine series and **10b** of the benzodioxine series) were selected for further analysis at various concentrations. Compound **1a4** ($K_{i\ (thrombin)}$ = 0.95 μM) caused a complete inhibition of angiogenesis and the degradation of immature, existing vessels at 250, 100 and 40 nmol/disc, and destruction of vessels with bleeding at 20 nmol/disc. Also **10b** ($K_{i\ (thrombin)}$ = 0.178 μM) completely abrogated CAM vascularization at 250 and 100 nmol/disc and vascular destruction at 40 nmol/disc. Only major, pre-existing mature blood vessels were not affected by the compounds (Fig. 4). Both compounds were more potent than the reference compound SU5416, the latter being toxic at 100 nmol/egg, and reduced angiogenesis by 47% at 40 nmol/disc.

In summary, esters of 1,4-benzoxazine and 1,4-benzodioxine series were found to be potent inhibitors of angiogenesis in CAM assay. However, since complete inhibition of angiogenesis was elicited by potent thrombin inhibitors such as **1b8** ($K_{i\ (thrombin)}$ = 18 nM) as well as by weak thrombin inhibitors such as **1a4** ($K_{i\ (thrombin)}$ = 0.95 μM) [23] a question arises as to whether other effects, besides thrombin inhibition, might contribute to their anti-angiogenic activity.

2.2.5. Radiometric protein kinase assay

In order to verify some of the best results obtained by docking to VEGFR2 variants, compounds **1a4** (R^2 = H) and **1a5** were tested for inhibition of VEGFR-2 kinase in a radiometric protein kinase assay in which inhibition of poly(Glu,Tyr) 4:1 substrate phosphorylation by isolated human recombinant VEGFR2 tyrosin kinase was measured [32]. Both compounds, screened as racemic mixtures, were found to be micromolar inhibitors with IC_{50} values of 22.5 μM for **1a4** (R^2 = H) and 80.0 μM for **1a5**. On the other side, it has been proven by IC_{50} determination that compound **1a4** (R^2 = H) inhibits VEGFR2 kinase in concentration depending manner [32]. From these results we can assume that, besides inhibiting of thrombin, the inhibition of angiogenesis observed by compounds **1** and **10** in the CAM assay, could to some extent also be due to slowing down of VEGFR2 activity.

3. Conclusion

In conclusion, 1,4-benzoxazine and 1,4-benzodioxine compounds **1** and **10**, which combine thrombin inhibitory and GPIIb/IIIa antagonistic activity in one molecule, were identified as potent inhibitors of angiogenesis in their ester form. The corresponding carboxylic acids were devoid of antiangiogenic activity, most probably due to their insufficient entry into the cell. Although thrombin inhibition remains the most probable explanation for their inhibition of angiogenesis by compounds **1** and **10**, other targets such as VEGFR2 might be involved. Future experiments should reveal the exact mechanism of action and potential anti-tumor and/or anti-metastatic activity of these compounds.

4. Experimental section

4.1. General

Chemicals were obtained from Acros, Aldrich Chemical Co. and Fluka and used without further purification. The synthesis of compound (*S*)-**10a** is described in ref. [23]b. Analytical TLC was performed on silica gel Merck 60 F_{254} plates (0.25 mm), using visualization with ultraviolet light. Column chromatography was carried out on silica gel 60 (particle size 240–400 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a 400 MHz Bruker AVANCE III spectrometer in DMSO-d_6 solution with TMS as the internal standard. The following abbreviations were used to describe peak patterns wherever appropriate: br = broad, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, and m = multiplet. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Microanalyses were performed on a Perkin-Elmer C, H, N Analyzer 240 C. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. HPLC Analyses were performed on an Agilent Technologies HP 1100 instrument with G1365B UV–VIS detector (254 nm), using an Eclips Plus C18 column (4.6 × 150 mm) at flow rate 1 mL/min. The eluent was a mixture of 0.1% TFA in water (A) and methanol (B). Gradient was 40% B to 80% B in 15 min. All of the compounds reported in this paper have a purity >95% (HPLC). Purifications of final esters by reverse phase column chromatography were performed using a Flash Purification System ISOLERA™. The eluent was a mixture of 0.1% TFA in water (A) and methanol (B). Gradient was 40% B to 80% B in 30 column volumes.

4.1.1. 4-((6-Nitro-2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy) benzonitrile (**6a**)

4-Cyanophenol (986 mg, 8.28 mmol) and triphenylphosphine (3.95 g, 15.06 mmol) were added to a solution of (6-nitro-2,3-dihydrobenzo[*b*] [1,4]dioxin-2-yl)methanol (**5a**) (1.59 g, 7.53 mmol) in anhydrous tetrahydrofurane (50 mL). Diisopropyl azodicarboxylate (DIAD) (3.05 g, 15.06 mmol) dissolved in 10 mL anhydrous THF was added dropwise at 0 °C, the solution was stirred afterward for 30 min at 0 °C, and then heated to reflux for 48 h. The reaction mixture was evaporated in vacuo to dryness and the residue purified by column chromatography on silica gel (hexane:ethyl acetate = 2:1). A yellow oil obtained was recrystallized from methanol to give 1.30 g (yield 55%) of pale yellow crystals; mp 171–174 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.33 (dd, *J* = 11.7, 7.3 Hz, 1H, 3-CH_2), 4.38 (dd, *J* = 11.1, 5.6 Hz, 1H, CH_2O), 4.45 (dd, *J* = 11.1, 3.7 Hz, 1H, CH_2O), 4.62 (dd, *J* = 11.7, 2.5 Hz, 1H, 3-CH_2), 4.72–4.78 (m, 1H, 2-CH), 7.15–7.28 (m, 3H, Ar–H^8, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.79–7.88 (m, 4H, Ar–H^5, Ar–H^7, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (101 MHz, DMSO-d_6): δ ppm 65.0 (C-3), 66.5 (CH_2O), 71.4 (C-2), 103.4 (C-4′), 112.7 (C-7), 115.7 (C-2′, C-6′), 117.5, 117.6 (C-5, C-8), 119.0 (CN), 134.2 (C-3′, C-5′), 141.2 (C-6), 142.4, 149.0 (C-4a, C-8a), 161.4 (C-1′); HRMS (ESI) *m/z* calcd for $C_{16}H_{13}N_2O_5$ $[M + H]^+$ 313.0824, found 313.0814; IR (KBr, *ν*, cm^{-1}): 2227, 1603, 1514, 1349, 1256, 1174, 839; HPLC: 100%, t_r 16.0 min.

4.1.2. 4-((7-Nitro-2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methoxy) benzonitrile (**6b**)

Compound **6b** was prepared from **5b** (1.59g, 7.53 mmol) and 4-cyanophenol (986 mg, 8.28 mmol) according to the procedure described above for the synthesis of **6a**; pale yellow crystals; yield 1.00 g (43%); mp 169–172 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.33 (dd, *J* = 11.7, 7.3 Hz, 1H, 3-CH_2), 4.38 (dd, *J* = 11.1, 5.7 Hz, 1H, CH_2O), 4.45 (dd, *J* = 11.1, 3.7 Hz, 1H, CH_2O), 4.62 (dd, *J* = 11.7, 2.5 Hz, 1H, 3-CH_2), 4.68–4.81 (m, 1H, 2-CH), 7.14–7.27 (m, 3H, Ar–H^5, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.76–7.86 (m, 4H, Ar–H^6, Ar–H^8, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (101 MHz, DMSO-d_6): δ ppm 64.9 (C-3), 66.5 (CH_2O), 71.4 (C-2), 103.4 (C-4′), 112.7 (C-6), 115.7 (C-2′, C-6′), 117.5, 117.6 (C-5, C-8), 119.0 (CN), 134.2 (C-3′, C-5′), 141.2 (C-7), 142.5, 149.0 (C-4a, C-8a), 161.4 (C-1′); HRMS (ESI) *m/z* calcd for $C_{16}H_{13}N_2O_5$ $[M + H]^+$ 313.0824, found 313.0828; IR (KBr, *ν*, cm^{-1}): 2225, 1600, 1504, 1349, 1251, 820; HPLC: 100%, t_r 16.0 min.

4.1.3. 4-((6-(Benzylamino)-2,3-dihydrobenzo[b][1,4]dioxin-2-yl) methoxy)benzonitrile (**8a**)

A mixture of compound **6a** (1.10 g, 3.52 mmol) and 10% Pd/C (110 mg) in methanol (100 mL) was stirred in a hydrogenator (25 bar) for 1 h at room temperature. The catalyst was filtered off and the solvent evaporated in vacuo to give amine **7a** (890 mg, 90%) of which was used in the next step without purification. The crude amine **7a** (890 mg, 3.15 mmol), benzaldehyde (334 mg, 3.15 mmol) and molecular sieves (3 Å) were mixed in methanol (50 mL) under Ar atmosphere and the mixture was stirred at room temperature for 12 h, until the aldimine formation was completed. The reaction mixture was carefully treated with solid $NaBH_4$ (191 mg, 5.04 mmol) and stirred for additional 1 h. After filtration and evaporation of solvent in vacuo, a crude residue was dissolved in dichloromethane (50 mL) and washed successively with saturated solution of $NaHCO_3$ (3 × 50 mL) and brine (1 × 50 mL). The organic solution was dried over Na_2SO_4 and the solvent evaporated under reduced pressure. The oily product was purified by column chromatography using dichloromethane as eluent to obtain 563 mg of **8a** as pale yellow crystals; yield 43% (from **6a**); mp 88–91 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.00 (dd, J = 11.4, 7.2 Hz, 1H, 3-CH_2), 4.18 (d, J = 5.9 Hz, 2H, PhCH_2), 4.18–4.31 (m, 3H, 3-CH_2, CH_2O), 4.40–4.51 (m, 1H, 2-CH), 5.93 (t, J = 5.9 Hz, 1H, NH), 6.07 (d, J = 2.6 Hz, 1H, Ar–H^5), 6.15 (dd, J = 8.7, 2.6 Hz, 1H, Ar–H^7), 6.62 (d, J = 8.7 Hz, 1H, Ar–H^8), 7.16 (d, J = 9.0 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.18–7.26 (m, 1H, Ph), 7.27–7.35 (m, 4H, Ph), 7.78 (d, J = 9.0 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (101 MHz, DMSO-d_6): δ ppm 47.0 (Ph-CH_2), 64.2 (C-3), 66.9 (CH_2O), 71.4 (C-2), 100.3 (C-5), 103.2 (C-4′), 106.3 (C-7), 115.6 (C-2′, C-6′), 117.1 (C-8), 119.0 (CN), 126.4 (C-4″), 127.1 (C-2″, C-6″), 128.2 (C-3″, C-5″), 133.6 (C-8a), 134.2 (C-3′, C-5′), 140.4 (C-6), 142.7 (C-1″), 143.9 (C-4a), 161.6 (C-1′); HRMS (ESI) *m/z* calcd for $C_{23}H_{21}N_2O_3$ $[M + H]^+$ 373.1552, found 373.1552; IR (KBr, *v*, cm^{-1}): 2219, 1606, 1504, 1260, 1174, 1045, 830; HPLC: 98.1%, t_r 11.7 min.

4.1.4. 4-((7-(Benzylamino)-2,3-dihydrobenzo[b][1,4]dioxin-2-yl) methoxy)benzonitrile (**8b**)

Starting from **6b** (1.10 g, 3.52 mmol) and benzaldehyde (334 mg, 3.15 mmol), compound **8b** was prepared according to the procedure described above for the synthesis of **8a**; yellow crystals, yield 539 mg (41% from **6b**); mp 115–118 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.00 (dd, J = 11.5, 7.2 Hz, 1H, 3-CH_2), 4.18 (d, J = 5.6 Hz, 2H, PhCH_2), 4.20–4.33 (m, 3H, 3-CH_2, CH_2O), 4.42–4.57 (m, 1H, 2-CH), 5.93 (t, J = 5.6 Hz, 1H, NH), 6.07 (d, J = 2.6 Hz, 1H, Ar–H^8), 6.15 (dd, J = 8.7, 2.6 Hz, 1H, Ar–H^6), 6.62 (d, J = 8.7 Hz, 1H, Ar–H^5), 7.16 (d, J = 9.0 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.19–7.25 (m, 1H, Ph), 7.27–7.38 (m, 4H, Ph), 7.78 (d, J = 9.0 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (101 MHz, DMSO-d_6): δ ppm 47.0 (Ph-CH_2) 64.2 (C-3), 66.7 (CH_2O), 71.4 (C-2), 100.3 (C-8), 103.2 (C-8″), 106.3 (C-6), 115.6 (C-2″, C-6′), 117.1 (C-5), 119.0 (CN), 126.5 (C-4″), 127.1 (C-2″, C-6″), 128.2 (C-3″, C-5″), 133.6 (C-4a), 134.2 (C-3′, C-5′), 140.4 (C-7), 142.7 (C-1″), 143.9 (C-8a), 161.6 (C-1′); HRMS (ESI) *m/z* calcd for $C_{23}H_{21}N_2O_3$ $[M + H]^+$ 373. 1552, found 373.1546; IR (KBr, *v*, cm^{-1}): 3387, 3050, 2220, 1508, 1262, 1174, 832; HPLC: 98.8%, t_r 11.7 min.

4.1.5. Ethyl 2-(benzyl(2-((4-cyanophenoxy)methyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl)amino)-2-oxoacetate (**9a**)

Ethyl oxalyl chloride (330 mg, 2.42 mmol) was added to a solution of **8a** (753 mg, 2.02 mmol) and triethylamine (245 mg, 2.42 mmol) in dichloromethane (50 mL) and the mixture stirred for 2 h. The solvent was removed under reduced pressure, the residue dissolved in ethyl acetate (50 mL) and washed successively with a 10% citric acid solution (3 × 50 mL), saturated $NaHCO_3$ solution (3 × 50 mL) and brine (1 × 50 mL). The organic phase was dried over Na_2SO_4 and the solvent evaporated under reduced pressure. The oily product was purified by column chromatography using dichloromethane as eluent to obtain 887 mg (93%) of **9a** as pale yellow amorphous solid; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 0.93 (t, J = 7.1 Hz, 3H, CH_2CH_3), 4.02 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.13 (dd, J = 11.6, 7.4 Hz, 1H, 3-CH_2), 4.30 (dd, J = 10.9, 5.7 Hz, 1H, CH_2O), 4.37 (dd, J = 10.9, 3.7 Hz, 1H, CH_2O), 4.44 (dd, J = 11.6, 2.4 Hz, 1H, 3-CH_2), 4.49–4.58 (m, 1H, 2-CH), 4.90 (s, 2H, PhCH_2), 6.62 (dd, J = 8.6, 2.7 Hz, 1H, Ar–H^7), 6.78 (d, J = 2.7 Hz, 1H, Ar–H^5), 6.89 (d, J = 8.6 Hz, 1H, Ar–H^8), 7.15 (d, J = 9.0 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.18–7.22 (m, 2H, Ph), 7.25–7.37 (m, 3H, Ph), 7.79 (d, J = 9.0 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (101 MHz, DMSO-d_6): δ ppm 13.4 (CH_2–CH_3), 50.8 (Ph-CH_2), 61.3 (CH_2–CH_3), 64.4 (C-3), 66.6 (CH_2O), 71.3 (C-2), 103.3 (C-4′), 115.6 (C-2′, C-6′), 116.2 (C-7), 117.4 (C-5), 119.0 (CN), 120.9 (C-8), 127.5 (C-4″), 128.0 (C-2″, C-6″), 128.6 (C-3″, C-5″), 132.3 (C-6), 134.2 (C-3′, C-5′), 136.1 (C-1′), 142.6, 142.7 (C-4a, C-8a), 161.4, 161.5, 162.4 (CO–COO, CO–COO, C-1′); HRMS (ESI) *m/z* calcd for $C_{27}H_{25}N_2O_6$ $[M + H]^+$ 473.1713, found 473.1711; IR (KBr, *v*, cm^{-1}): 2224, 1605, 1507, 1252, 1175, 1016, 834; HPLC: 100%, t_r 17.6 min.

4.1.6. Ethyl 2-(benzyl(3-((4-cyanophenoxy)methyl)-2,3-dihydrobenzo [b][1,4]dioxin-7-yl)amino)-2-oxoacetate (**9b**)

Compound **9b** was prepared from **8b** (753 mg, 2.02 mmol) and ethyl oxalyl chloride (330 mg, 2.42 mmol) according to the procedure described above for the synthesis of **9a**; pale yellow solid, yield 811 mg (85%); ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 0.93 (t, J = 7.1 Hz, 3H, CH_2CH_3), 4.01 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.11 (dd, J = 11.6, 7.4 Hz, 1H, 3-CH_2), 4.30 (dd, J = 11.1, 5.8 Hz, 1H, CH_2O), 4.37 (dd, J = 11.1, 3.7 Hz, 1H, CH_2O), 4.44 (dd, J = 11.6, 2.4 Hz, 1H, 3-CH_2), 4.56–4.64 (m, 1H, 2-CH), 4.90 (s, 2H, PhCH_2), 6.62 (dd, J = 8.6, 2.5 Hz, 1 = , Ar–H^6), 6.78 (d, J = 2.5 Hz, 1H, Ar–H^8), 6.89 (d, J = 8.6 Hz, 1H, Ar–H^5), 7.15 (d, J = 9.0 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.18–7.24 (m, 2H, Ph), 7.25–7.38 (m, 3H, Ph), 7.79 (d, J = 9.0 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (DMSO-d_6) δ ppm: 13.4 (CH_2–CH_3), 50.8 (Ph-CH_2), 61.3 (CH_2–CH_3), 64.4 (C-3), 66.6 (CH_2O), 71.3 (C-2), 103.3 (C-4′), 115.6 (C-2′, C-6′), 116.1 (C-6), 117.4 (C-5), 119.0 (CN), 120.9 (C-8), 127.5 (C-4″), 128.0 (C-2″, C-6″), 128.6 (C-3″, C-5″), 132.3 (C-7), 134.2 (C-3′, C-5′), 136.1 (C-1″), 142.6, 142.7 (C-4a, C-8a), 161.4, 161.5, 162.4 (CO–COO, CO–COO, C-1′); HRMS (ESI) *m/z* calcd for $C_{27}H_{25}N_2O_6$ $[M + H]^+$ 473.1713, found 473.1725; IR (KBr, *v*, cm^{-1}): 2225, 1741, 1670, 1606, 1508, 1256, 1172, 1029, 835; HPLC: 100%, t_r 17.5 min.

4.1.7. Ethyl 2-(benzyl(2-((4-carbamimidoylphenoxy)methyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl)amino)-2-oxoacetate trifluoroacetate (**10a**)

Gaseous HCl was slowly introduced over 30 min into a solution of the nitrile **9a** (482 mg, 1.02 mmol) in anhydrous ethanol (30 mL). The reaction mixture was closed tightly and stirred for 24 h at room temperature. The solvent was evaporated in vacuo and the residue washed 3 times with anhydrous diethyl ether. The iminoether obtained was dissolved in anhydrous EtOH (30 mL), ammonium acetate (236 mg, 3.06 mmol) was added and the reaction mixture stirred for 24 h at room temperature. The solvent was evaporated in vacuo and the crude product purified by gradient reverse phase column chromatography using methanol/trifluoroacetic acid (40–80% in 30 min) as eluent. After evaporation of volatiles, white crystals were precipitated from trifluoroacetic acid, filtered off and dried to yield 307 mg (50%) of **10a** as a white powder; mp 192–194 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 0.93 (t, J = 7.1 Hz, 3H, CH_2CH_3), 4.02 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.14 (dd, J = 11.7, 7.4 Hz, 1H, 3-CH_2), 4.32 (dd, J = 11.0, 5.7 Hz, 1H, CH_2O), 4.39 (dd, J = 10.9, 3.6 Hz, 1H, CH_2O), 4.46 (dd, J = 11.6, 2.4 Hz, 1H, 3-CH_2), 4.57–4.66 (m, 1H, 2-CH), 4.91 (s, 2H, PhCH_2), 6.63 (dd, J = 8.6, 2.7 Hz, 1H, Ar–H^8), 6.79 (d, J = 2.7 Hz, 1H, Ar–H^7), 6.89

(d, J = 8.6 Hz, 1H, Ar–H^5), 7.16–7.25 (m, 4H, Ph, Ar–H$^{2'}$, Ar–H$^{6'}$), 7.27–7.37 (m, 3H, Ph), 7.83 (d, J = 9.0 Hz, 2H, Ar–H$^{3'}$, Ar–H$^{5'}$), 8.92 (s, 2H, NH_2), 9.16 (s, 2H, NH_2^+); ^{13}C NMR (101 MHz, DMSO-d_6): δ ppm 13.4 (CH_2–$\underline{CH_3}$), 50.8 (Ph-$\underline{CH_2}$), 61.3 ($\underline{CH_2}$–CH_3), 64.4 (C-3), 66.7 (CH_2O), 71.3 (C-2), 114.8 (C-2′, C-6′), 116.2 (C-7), 117.1 ($\underline{CF_3}$–COOH, $^1J_{C,F}$ = 299.3 Hz), 117.4 (C-5), 120.1 (C-4′), 120.9 (C-8), 127.6 (C-4″), 128.0 (C-2″, C-6″), 128.6 (C-3″, C-5″), 130.2 (C-3′, C-5′), 132.3 (C-6), 136.1 (C-1″), 142.6, 142.7 (C-4a, C-8a), 158.7 (CF_3–$\underline{COOH}$, $^2J_{C,F}$ = 31.5 Hz), 161.5, 162.3, 162.4, 164.5 ($\underline{CO}$–COO, CO–$\underline{CO}$O, C-1′, C(=NH)NH_2); HRMS (ESI) m/z calcd for $C_{27}H_{28}N_3O_6$ [M + H]$^+$ 490.1978, found 490.1972; IR (KBr, ν, cm^{-1}): 3298, 3122, 1737, 1665, 1506, 1203, 843; HPLC: 100%, t_r 12.4 min.

4.1.8. Ethyl 2-(benzyl(3-((4-carbamimidoylphenoxy)methyl)-2,3-dihydrobenzo[b][1,4]dioxin-7-yl)amino)-2-oxoacetate trifluoroacetate (**10b**)

Compound **10b** was prepared from nitrile **9b** (482 mg, 1.02 mmol) according to procedure described above for the synthesis of **10a**; white powder, yield 335 mg (54%); mp 185–188 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 0.91 (t, J = 7.1 Hz, 3H, $CH_2\underline{CH_3}$), 4.00 (q, J = 7.1 Hz, 2H, $\underline{CH_2}CH_3$), 4.15 (dd, J = 11.6, 7.2 Hz, 1H, 3-$\underline{CH_2}$), 4.31 (dd, J = 11.1, 6.1 Hz, 1H, CH_2O), 4.39 (dd, J = 10.9, 3.4 Hz, 1H, CH_2O), 4.46 (dd, J = 11.6, 2.2 Hz, 1H, 3-CH_2), 4.58–4.64 (m, 1H, 2-CH), 4.90 (s, 2H, Ph$\underline{CH_2}$), 6.63 (dd, J = 8.7, 2.5 Hz, 1H, Ar–H^6), 6.80 (d, J = 2.5 Hz, 1H, Ar–H^8), 6.89 (d, J = 8.7 Hz, 1H, Ar–H^5), 7.15–7.38 (m, 7H, Ph, Ar–H$^{2'}$, Ar–H$^{6'}$), 7.83 (d, J = 8.9 Hz, 2H, Ar–H$^{3'}$, Ar–H$^{5'}$), 8.91 (s, 2H, NH_2), 9.17 (s, 2H, NH_2^+); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm: 13.3 (CH_2–$\underline{CH_3}$), 50.8 (Ph-$\underline{CH_2}$), 61.3 ($\underline{CH_2}$–CH_3), 64.4 (C-3), 66.7 (CH_2O), 71.3 (C-2), 114.8 (C-2′, C-6′), 116.2 (C-6), 117.1 ($\underline{CF_3}$–COOH, $^1J_{C,F}$ = 299.2 Hz), 117.3 (C-5), 120.1 (C-4′), 120.8 (C-8), 127.5 (C-4″), 127.9 (C-2″, C-6″), 128.6 (C-3″, C-5″), 130.2 (C-3′, C-5′), 132.5 (C-7), 136.1 (C-1″), 142.5, 142.8 (C-4a, C-8a), 158.8 (CF_3–$\underline{COOH}$, $^2J_{C,F}$ = 31.5 Hz), 161.5, 162.3, 162.4, 164.5 ($\underline{CO}$–COO, CO–$\underline{CO}$O, C-1′, C(=NH)NH_2); HRMS (ESI) m/z calcd for $C_{27}H_{28}N_3O_6$ [M + H]$^+$ 490.1978, found 490.1970; IR (KBr, ν, cm^{-1}): 3345, 3109, 1742, 1670, 1500, 1197, 843; HPLC: 98.4%, t_r 12.7 min.

4.1.9. 2-(Benzyl(2-((4-carbamimidoylphenoxy)methyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl)amino)-2-oxoacetic acid (**11a**)

To a solution of ester **10a** (150 mg, 0.25 mmol) in tetrahydrofuran (3 mL) and methanol (1 mL), 1 M LiOH (1.50 mL, 1.50 mmol) was added and the mixture was stirred for 1 h at room temperature. The organic solvents were evaporated under vacuum and the resulting aqueous solution neutralized with 0.1% trifluoroacetic acid to precipitate the product which was filtered off and dried to obtain 72 mg (63%) of **11** as a white powder, mp 290–293 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.14 (dd, J = 11.6, 7.6 Hz, 1H, 3-CH_2), 4.33 (dd, J = 11.0, 5.6 Hz, 1H, CH_2O), 4.40 (dd, J = 11.0, 3.7 Hz, 1H, CH_2O), 4.46 (dd, J = 11.6, 2.3 Hz, 1H, 3-CH_2), 4.57–4.66 (m, 1H, 2-CH), 4.88 (s, 2H, Ph$\underline{CH_2}$), 6.67 (dd, J = 8.6, 2.5 Hz, 1H, Ar–H^7), 6.80 (d, J = 2.5 Hz, 1H, Ar–H^5), 6.89 (d, J = 8.6 Hz, 1H, Ar–H^8), 7.18–7.24 (m, 4H, Ph, Ar–H$^{2'}$, Ar–H$^{6'}$), 7.27–7.35 (m, 3H, Ph), 7.83 (d, J = 8.9 Hz, 2H, Ar–H$^{3'}$, Ar–H$^{5'}$), 9.04 (s, 2H, NH_2), 9.17 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm: 49.6 (Ph-$\underline{CH_2}$), 64.4 (C-3), 66.7 (CH_2O), 71.0 (C-2), 114.7 (C-2′, C-6′), 115.9 (C-7), 116.6 (C-5), 120.2 (C-4′), 120.6 (C-8), 127.0 (C-4″), 127.6 (C-2″, C-6″), 128.3 (C-3″, C-5″), 129.8 (C-3′, C-5′), 132.3 (C-6), 137.7 (C-1″), 141.4, 142.0 (C-4a, C-8a), 162.0, 163.2, 164.0, 164.6 ($\underline{CO}$–COO, CO–$\underline{CO}$O, C-1′, C(=NH)NH_2); HRMS (ESI) m/z calcd for $C_{25}H_{24}N_3O_6$ [M + H]$^+$ 462.1665, found 462.1677; IR (KBr, ν, cm^{-1}): 3368, 1609, 1503, 1255, 844; HPLC: 96.1%, t_r 9.5 min.

4.1.10. 2-(Benzyl(3-((4-carbamimidoylphenoxy)methyl)-2,3-dihydrobenzo[b][1,4]dioxin-7-yl)amino)-2-oxoacetic acid (**11b**)

Compound **11b** was prepared from **10b** (150 mg, 0.25 mmol) according to procedure described above for the synthesis of **11a**; white powder, yield 69 mg (60%); mp 291–294 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.13 (dd, J = 11.6, 7.9 Hz, 1H, 3-CH_2), 4.34 (dd, J = 10.9, 5.6 Hz, 1H, CH_2O), 4.40 (dd, J = 10.9, 3.6 Hz, 1H, CH_2O), 4.48 (dd, J = 11.6, 2.2 Hz, 1H, 3-CH_2), 4.55–4.65 (m, 1H, 2-CH), 4.88 (s, 2H, Ph$\underline{CH_2}$), 6.68 (dd, J = 8.6, 2.5 Hz, 1H, Ar–H^6), 6.80 (d, J = 2.5 Hz, 1H, Ar–H^8), 6.89 (d, J = 8.6 Hz, 1H, Ar–H^5), 7.18–7.37 (m, 7H, Ph, Ar–H$^{2'}$, Ar–H$^{6'}$), 7.83 (d, J = 8.9 Hz, 2H, Ar–H$^{3'}$, Ar–H$^{5'}$), 8.91 (s, 2H, NH_2), 9.17 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm: 50.6 (Ph-$\underline{CH_2}$), 64.4 (C-3), 66.8 (CH_2O), 71.4 (C-2), 114.9 (C-2′, C-6′), 116.1 (C-8), 117.3 (C-6), 120.1 (C-4′), 120.7 (C-5), 127.4 (C-4″), 127.8 (C-2″, C-6″), 128.5 (C-3″, C-5″), 130.2 (C-3′, C-5′), 133.0 (C-7), 136.3 (C-1″), 142.5, 142.6 (C-4a, C-8a), 162.3, 163.1, 164.2, 164.5 ($\underline{CO}$–COO, CO–$\underline{CO}$O, C-1′, C(=NH)NH_2); HRMS (ESI) m/z calcd for $C_{25}H_{24}N_3O_6$ [M + H]$^+$ 462.1665, found 462.1674; IR (KBr, ν, cm^{-1}): 3309, 1609, 1492, 1262, 839; HPLC: 96.2%, t_r 9.7 min.

4.1.11. (2,4-Dimethyl-7-nitro-3,4-dihydro-2H-benzo[b][1,4]oxazin-2-yl)methyl acetate (**13**)

Alcohol **12** [22] (2.00 g, 8.4 mmol) was dissolved in acetic anhydride (50 mL) and heated at 100 °C for 5 h. The excess acetic anhydride was removed in vacuo to yield 2.02 g (86%) of **13** as brown oil; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.36 (s, 3H, 2-CH_3), 2.09 (s, 3H, $COCH_3$), 2.99 (s, 3H, NCH_3), 3.16 (d, J = 12.1 Hz, 1H, 3-H), 3.32 (d, J = 12.1 Hz, 1H, 3-H), 4.15 (d, J = 11.6 Hz, 1H, CH_2O), 4.20 (d, J = 11.6 Hz, 1H, CH_2O), 6.92 (d, J = 8.8 Hz, 1H, Ar–H^5), 7.51 (d, J = 2.6 Hz, 1H, Ar–H^8), 7.58 (dd, J = 8.8, 2.6 Hz, 1H, Ar–H^6); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 20.5 (CO$\underline{CH_3}$), 20.6 (2-CH_3), 38.0 (NCH_3), 52.8 (C-3), 66.0 ($\underline{CH_2}$O), 75.4 (C-2), 106.2 (C-8), 114.1 (C-6), 115.7 (C-5), 135.4 (C-7), 141.5 (C-8a), 148.2 (C-4a), 170.0 (CO); HRMS (ESI) m/z calcd for $C_{13}H_{17}N_2O_5$ [M + H]$^+$ 281.1137, found 281.1132; HPLC: 97.0%, t_r 14.3 min; Anal. ($C_{13}H_{16}N_2O_5$): C, H, N.

4.1.12. (7-(Benzylamino)-2,4-dimethyl-3,4-dihydro-2H-benzo[b][1,4]oxazin-2-yl)methanol (**15a**)

Prepared from compound **13** (1.66 g, 5.93 mmol) and benzaldehyde (548 mg, 5.16 mmol) following the procedure for the synthesis of compound **8a**; yellow oil, yield 1.23 g (70% from **13**); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.16 (s, 3H, 2-CH_3), 2.74 (s, 3H, NCH_3), 2.81 (d, J = 11.4 Hz, 1H, 3-H), 3.03 (d, J = 11.4 Hz, 1H, 3-H), 3.27 (dd, J = 10.7, 5.8 Hz, 1H, CH_2O), 3.35–3.43 (m, overlapped with H_2O, 1H, CH_2O), 4.18 (d, 6.13 Hz, N–CH_2), 4.90 (t, J = 5.3 Hz, 1H, OH), 5.56 (t, J = 5.6 Hz, 1H, NH), 5.83 (dd, J = 8.4, 2.3 Hz, 1H, Ar–H^6), 6.03 (d, J = 2.3 Hz, 1H, Ar–H^8), 6.37 (d, J = 8.4 Hz, 1H, Ar–H^5), 7.17–7.40 (m, 5H, Ph); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 20.9 (2-CH_3), 38.3 (NCH_3), 47.5 (NCH_2), 54.2 (C-3), 65.0 (CH_2O), 74.5 (C-2), 97.6 (C-6), 101.8 (C-8), 115.5 (C-5), 126.4 (C-4′), 127.2 (C-2′, C-6′), 128.1 (C-3′, C-5′), 134.4, 135.5 (C-4a, C-8a), 140.9, 143.1 (C-7, C-1′); HRMS (ESI) m/z calcd for $C_{18}H_{23}N_2O_2$ [M + H]$^+$ 299.1760, found 299.1755; HPLC: 97.3%, t_r 6.5 min; Anal. ($C_{18}H_{22}N_2O_2 \times 1/4H_2O$): C, H, N.

4.1.13. (7-((3,5-Difluorobenzyl)amino)-2,4-dimethyl-3,4-dihydro-2H-benzo[b][1,4]oxazin-2-yl)methanol (**15b**)

Prepared from compound 13 (1.71 g, 6.10 mmol) and 3,5-difluorobenzaldehyde (693 mg, 4.88 mmol) following the procedure for the synthesis of compound **8a**; yellow oil, yield 1.19 g (58% from **13**); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.16 (s, 3H, 2-CH_3), 2.75 (s, 3H, NCH_3), 2.82 (d, J = 11.36 Hz, 1H, 3-H), 3.04 (d, J = 11.36 Hz, 1H, 3-H), 3.28 (dd, J = 10.59, 5.16 Hz, 1H, CH_2O), 3.42–3.36 (m, 1H, CH_2O), 4.22 (d, 6.13 Hz, N–CH_2), 4.91 (t, J = 5.3 Hz, 1H, OH), 5.48 (t, J = 5.6 Hz, 1H, NH), 5.79 (dd, J = 8.38, 1.79 Hz, 1H, Ar–H^6), 6.01 (d, J = 1.68 Hz, 1H Ar–H^8), 6.38 (d, J = 8.40 Hz, 1H, Ar–H^5), 6.99–7.12 (m, 3H, 3Ar–H); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 20.9 (2-CH_3), 38.2 (NCH_3), 46.6 (CH_2N), 54.2 (C-3), 64.9 (CH_2O), 74.5 (C-2), 97.7 (C-6), 101.7 (C-8), 101.7 (t, $^2J_{C-F}$ = 25.9 Hz, C-4′), 109.5 (dd, $^2J_{C-F}$ = 24.7 Hz, $^4J_{C-F}$ = 6.3 Hz, C-2′, C-6′), 115.6 (C-5), 134.7,

135.6, 142.4 (C-7, C-4a, C-8a), 146.6 (t, $^3J_{C-F}$ = 8.2 Hz, C-1′), 162.4 (dd, $^1J_{C-F}$ = 245.8 Hz, $^3J_{C-F}$ = 13.1 Hz, C-3′, C-5′); HRMS (ESI) *m/z* calcd for $C_{18}H_{21}N_2O_2F_2$ $[M + H]^+$ 335.1571, found 335.1558; HPLC: 96.9%, t_r 7.8 min; Anal. ($C_{18}H_{20}N_2O_2F_2$): C, H, N.

4.1.14. Ethyl 2-(benzyl(2-(hydroxymethyl)-2,4-dimethyl-3,4-dihydro-2H-benzo[b][1,4]oxazin-7-yl)amino)-2-oxoacetate (**16a**)

Compound **16a** was prepared from **15a** (483 mg, 1.62 mmol) and ethyl oxalyl chloride (221 mg, 1.62 mmol) according to the procedure described above for the synthesis of **9a**; yellow oil, yield 458 mg (71%); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.89 (t, J = 7.1 Hz, 3H, $CH_2\underline{CH_3}$), 1.17 (s, 3H, 2-CH_3), 2.82 (s, 3H, NCH_3), 2.89 (d, J = 11.7 Hz, 1H, 3-H), 3.11 (d, J = 11.7 Hz, 1H, 3-H), 3.29 (dd, J = 10.8, 5.9 Hz, 1H, CH_2O), 3.33–3.43 (m, overlapped with H_2O, 1H, CH_2O), 3.97 (q, J = 7.1 Hz, 1H, $\underline{CH_2}CH_3$), 4.85 (s, 2H, CH_2N), 5.04 (t, J = 5.7 Hz, 1H, OH), 6.48 (d, J = 2.2 Hz, 1H, Ar–H^8), 6.51 (dd, J = 8.5, 2.2 Hz, 1H, Ar–H^6), 6.60 (d, J = 8.5 Hz, 1H, Ar–H^5), 7.19 (d, J = 7.4 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.24–7.30 (m, 1H, Ar–$H^{4'}$), 7.33 (m, J = 7.4 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 13.4 ($CH_2\underline{CH_3}$), 20.7 (2-CH_3), 38.1 (NCH_3), 50.9 (CH_2N), 53.2 (C-3), 61.0 ($\underline{CH_2}CH_3$), 64.7 (CH_2O), 75.8 (C-2), 111.3 (C-5), 114.5 (C-8), 120.0 (C-6), 127.4 (C-4′), 127.8 (C-2′, C-6′), 128.5 (C-3′, C-5′), 128.7 (C-7), 135.5, 136.4, 142.5 (C-1′, C-4a, C-8a), 161.8, 162.7 ($\underline{CO}$–COO, CO–$\underline{COO}$); HRMS (ESI) *m/z* calcd for $C_{22}H_{27}N_2O_5$ $[M + H]^+$ 399.1920, found 399.1916; HPLC: 96.8%, t_r 14.5 min; Anal. ($C_{22}H_{26}N_2O_5$): C, H, N.

4.1.15. Ethyl 2-((3,5-difluorobenzyl)(2-(hydroxymethyl)-2,4-dimethyl-3,4-dihydro-2H-benzo[b][1,4]oxazin-7-yl)amino)-2-oxoacetate (**16b**)

Compound **16b** was prepared from **15b** (592 mg, 1.77 mmol) and ethyl oxalyl chloride (242 mg, 1.77 mmol) according to the procedure described above for the synthesis of **9a**; yellow oil, yield 529 mg (69%); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.90 (t, J = 7.1 Hz, 3H, $CH_2\underline{CH_3}$), 1.18 (s, 3H, 2-CH_3), 2.83 (s, 3H, NCH_3), 2.90 (d, J = 11.7 Hz, 1H, 3-H), 3.12 (d, J = 11.7 Hz, 1H, 3-H), 3.30 (dd, J = 10.8, 4.1 Hz, 1H, CH_2O), 3.40 (dd, J = 10.8, 4.1 Hz, 1H, CH_2O), 3.99 (d, J = 7.1 Hz, 1H, $\underline{CH_2}CH_3$), 4.88 (s, 2H, CH_2N), 5.04 (t, J = 5.7 Hz, 1H, OH), 6.52–6.57 (m, 2H, Ar–H^8, Ar–H^6), 6.63 (d, J = 8.3 Hz, 1H, Ar–H^5), 6.87–6.94 (m, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.13–7.22 (m, 1H, Ar–$H^{4'}$); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 13.4 ($CH_2\underline{CH_3}$), 20.7 (2-CH_3), 38.1 (NCH_3), 50.2 (CH_2N), 53.2 (C-3), 61.2 ($\underline{CH_2}CH_3$), 64.7 (CH_2O), 75.8 (C-2), 103.0 (t, $^2J_{C-F}$ = 25.8 Hz, C-4′), 110.9 (dd, $^2J_{C-F}$ = 18.6 Hz, $^4J_{C-F}$ = 6.8 Hz, C-2′, C-6′), 111.4 (C-5), 114.2 (C-8), 119.8 (C-6), 128.4 (C-7), 135.7 (C-8a), 141.1 (t, $^3J_{C-F}$ = 9.1 Hz, C-1′), 142.6 (C-4a), 161.9 ($\underline{CO}$–COO), 162.3 (dd, $^1J_{C-F}$ = 246.7 Hz, $^3J_{C-F}$ = 13.2 Hz, C-3′, C-5′), 162.5 (CO–$\underline{COO}$); HRMS (ESI) *m/z* calcd for $C_{22}H_{25}N_2O_5F_2$ $[M + H]^+$ 435.1732, found 435.1738; HPLC: 97.6%, t_r 15.6 min; Anal. ($C_{22}H_{24}N_2O_5F_2$): C, H, N.

4.1.16. 2-(Benzyl(2-(hydroxymethyl)-2,4-dimethyl-3,4-dihydro-2H-benzo[b][1,4]oxazin-7-yl)amino)-2-oxoacetic acid (**17a**)

Compound **17a** was prepared by hydrolysis of ester **16a** (398 mg, 1 mmol) with 1 M LiOH (6 mL) in a mixture of tetrahydrofuran and methanol according to the procedure for the synthesis of compound **11a** to obtain 296 mg (78%) of **17a** as brown oil; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.18 (s, 3H, 2-CH_3), 2.82 (s, 3H, NCH_3), 2.89 (d, J = 11.6 Hz, 1H, 3-H), 3.11 (d, J = 11.6 Hz, 1H, 3-H), 3.30 (d, J = 10.8 Hz, 1H, CH_2O), 3.40 (d, J = 10.8 Hz, 1H, CH_2O), 4.83 (s, 2H, CH_2N), 5.04 (bs, 1H, OH), 6.48–6.64 (m, 3H, Ar–H^8, Ar–H^6, Ar–H^5), 7.20 (d, J = 7.5 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.23–7.29 (m, 1H, Ar–$H^{4'}$), 7.32 (d, J = 7.5 Hz, 2H, Ar–$H^{3'}$, Ar–$H^{5'}$); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 20.8 (2-CH_3), 38.1 (NCH_3), 50.8 (CH_2N), 53.2 (C-3), 64.8 (CH_2O), 75.9 (C-2), 111.4 (C-5), 114.3 (C-8), 119.9 (C-6), 127.3 (C-4′), 127.7 (C-2′, C-6′), 128.4 (C-3′, C-5′), 129.3 (C-7), 135.3, 136.7, 142.6 (C-1′, C-4a, C-8a), 163.3, 164.4 ($\underline{CO}$–COO, CO–$\underline{COO}$); HRMS (ESI) *m/z* calcd for $C_{20}H_{23}N_2O_5$ $[M + H]^+$ 371.1607, found 371.1594; HPLC: 100%, t_r 11.3 min; Anal. ($C_{20}H_{22}N_2O_5$): C, H, N.

4.1.17. 2-((3,5-Difluorobenzyl)(2-(hydroxymethyl)-2,4-dimethyl-3,4-dihydro-2H-benzo[b][1,4]oxazin-7-yl)amino)-2-oxoacetic acid (**17b**)

Compound **17b** was prepared by hydrolysis of ester **16b** (434 mg, 1 mmol) with 1 M LiOH (6 mL) in a mixture of tetrahydrofuran and methanol according to the procedure for the synthesis of compound **11a** to obtain 292 mg (72%) of **17b** as brown oil; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.19 (s, 3H, 2-CH_3), 2.83 (s, 3H, NCH_3), 2.90 (d, J = 11.7 Hz, 1H, 3-H), 3.12 (d, J = 11.7 Hz, 1H, 3-H), 3.31 (d, J = 10.8 Hz, 1H, CH_2O), 3.41 (d, J = 10.8 Hz, 1H, CH_2O), 4.86 (s, 2H, CH_2N), 5.05 (bs, 1H, OH), 6.53–6.66 (m, 3H, Ar–H^8, Ar–H^6, Ar–H^5), 6.92 (d, J = 6.6 Hz, 2H, Ar–$H^{2'}$, Ar–$H^{6'}$), 7.04–7.18 (m, 1H, Ar–$H^{4'}$); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 20.8 (2-CH_3), 38.1 (NCH_3), 50.1 (CH_2N), 53.2 (C-3), 65.0 (CH_2O), 76.0 (C-2), 102.9 (t, $^2J_{C-F}$ = 25.8 Hz, C-4′), 110.7 (dd, $^2J_{C-F}$ = 18.6 Hz, $^4J_{C-F}$ = 6.8 Hz, C-2′, C-6′), 111.5 (C-5), 114.1 (C-8), 119.7 (C-6), 129.0 (C-7), 135.4 (C-8a), 141.4 (t, $^3J_{C-F}$ = 8.9 Hz, C-1′), 142.6 (C-4a), 162.4 (dd, $^1J_{C-F}$ = 246.5 Hz, $^3J_{C-F}$ = 13.2 Hz, C-3′, C-5′), 163.4 ($\underline{CO}$–COO), 164, 2 (CO–$\underline{COO}$); HRMS (ESI) *m/z* calcd for $C_{20}H_{21}N_2O_5F_2$ $[M + H]^+$ 407.1419, found 407.1414; HPLC: 93.0%, t_r 12.4 min; Anal. ($C_{20}H_{20}N_2O_5F_2$): C, H, N.

4.1.18. Cell cultures

Bovine aortic endothelial cells (BAEC) were kindly provided by Prof. M. Presta (Brescia, Italy). Human cervical carcinoma (HELA) and human breast carcinoma (MCF-7) cells were obtained from the American Type Culture Collection (ATCC, Middlesex, UK). The cells were grown in Dulbecco's modified minimum essential medium (DMEM, Life Technologies, Inc., Rockville, MD) supplemented with 10 mM Hepes (Life Technologies, Inc., Rockville, MD) and 10% fetal bovine serum (FBS, Harlan Sera-Lab Ltd., Loughborough, UK). Human microvascular endothelial cells (HMEC-1) were obtained from the Centers of Disease Control (CDC, Atlanta, GA) and grown in EGM-2 medium with supplements and growth factors (Lonza, Verviers, Belgium).

4.1.19. Cell proliferation assays

Cells (HMEC-1, BAEC, HELA or MCF-7) were seeded in 48-well plates at 10,000 cells per cm^2. After 16 h, the cells were incubated in fresh medium in the presence of the test compounds, as indicated in the Results section. On day 4, (BAEC, HELA, MCF-7) or day 7 (HMEC-1) cells were trypsinized and counted by means of a Coulter counter (Analis, Belgium). For each compound, the IC_{50} value was determined. This is the concentration of compound that causes 50% inhibition of cell proliferation.

4.1.20. Cell migration assay

Wounds were created in confluent MAEC monolayers with a 1.0-mm wide micropipette tip. Then, cells were incubated in fresh medium in the presence of the test compounds. After 5 h, the wounds were photographed and the width of the wound was calculated from digital pictures. For statistical analyses, the p values were determined using the Student t test, and p values < 0.05 were considered significant.

4.1.21. Tube formation assay

Wells of a 96-well plate were coated with 60 μl matrigel (10 mg/mL, BD Biosciences, Heidelberg, Germany) at 4 °C. After gelatinization at 37 °C during 30 min, HMEC-1 (60,000 cells) were seeded on top of the matrigel in 200 μl DMEM containing the test compounds. After 4–6 h of incubation, the cells were photographed and tube formation was quantified by giving a score from 0 (no

tubes) to 3 (maximal tube formation, comparable to non-treated control cultures). Statistical analysis was performed by using the Student *t* test, and *p* values < 0.05 were considered significant.

4.1.22. CAM assay

Fertilized eggs were incubated for 3 days at 37 °C when 3 mL of albumen was removed (to detach the shell from the developing CAM) and a window was opened on the eggshell exposing the CAM. The window was covered with cellophane tape and the eggs were returned to the incubator until day 9 when the compounds were applied. The compounds were placed on sterile plastic discs (Ø 8 mm), which were allowed to dry under sterile conditions. A solution of cortisone acetate (100 lg/disc, Sigma, St. Louis, MO) was added to all discs in order to prevent an inflammatory response. A loaded and dried control disc was placed on the CAM approximately 1 cm away from the disc containing the test compound(s). Next, the windows were covered and the eggs further incubated until day 11 when the area around the discs was cut-off and photographed. Next 2 concentric circles were positioned on the digitalized pictures and all vessels intersecting these circles were counted. Statistical analysis was performed by using the Student *t* test, and *p* values < 0.05 were considered significant.

4.1.23. In vitro VEGFR2 kinase assay

A radiometric protein kinase assay (^{33}PanQinase® Activity Assay) was used for measuring the kinase activity of the VEGFR2 protein kinase. VEGFR2 tyrosine kinase was expressed in Sf9 insect cells as human recombinant GST-fusion protein. The kinase was purified by affinity chromatography using GSH-agarose. The purity of the kinase was checked by SDS-PAGE/silver staining and the identity of the kinase was verified by mass spectroscopy. The IC_{50} profile of two compounds **1a4** and **1a5** (both in racemic form as trifluoroacetate salts) was determined. IC_{50} values were measured by testing 10 concentrations (1×10^{-4} M to 3×10^{-9} M) of each compound. The measurements have been performed by ProQinase company in singlicate [32].

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